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Applicant : Frederick M. BOYCE — Art Unit: 1819
Serial No.: 08/752,032 — Examiner: A. Razzaque
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Title : USE OF A BACULOVIRUS TO EXPRESS AN EXOGENOUS GENE IN A MAMMALIAN CELL

Assistant Commissioner for Patents
Washington, DC 20231

DECLARATION OF FREDERICK M. BOYCE UNDER 37 C.F.R. § 1.132

1. I am the inventor of the subject matter claimed in U.S. Serial No. 08/752,032 (hereinafter, the '032 application).

2. In an Office Action dated May 8, 1997, claims 1, 19, and 21-25 of the '032 application were rejected for lack of enablement on the ground that the specification does not teach how to express an exogenous gene *in vivo* such the infected cell will be alive and provide treatment of a gene deficiency disease. The experiments described below demonstrate that, as was asserted in the '032 application, the methods disclosed in the application can be used to express an exogenous gene in a variety of mammalian cells *in vitro* or *in vivo*. This Declaration summarizes the following experiments:

I. Expression of an exogenous gene in 15 of 19 mammalian cell types *in vitro*.

II. Expression of an exogenous gene in neuronal and glial cells of rat cerebral cortex cultures.

III. Exogenous gene expression *in vivo*.

IV. Expression of a therapeutic gene in human cells.

All of these experiments were carried out essentially as described in the '032 application. Cell types that expressed the exogenous gene include cells derived from: HepG2, 293, PC12 (treated with Nerve Growth Factor (NGF)), HeLa, CHO/dhfr$^-$, C_2C_{12} myoblasts, C_2C_{12} myotubes, primary human myoblasts, Sk-Hep-1, NIH3T3, NIH3T3 (expressing an asialoglycoprotein receptor (ASGPR)), Hep3B, FTO2B, Hepa 1-6 cells, neurons, glia, skin, spleen, kidney, stomach, skeletal muscle, uterus, and pancreas.

I. Expression of an Exogenous Gene in 15 of 19 Mammalian Cell Types

In the following examples, the ability of the Z4 baculovirus to direct exogenous gene expression in 19 different types of cells was tested. The Z4 virus was prepared by homologous recombination of the Z4 transfer plasmid with linearized AcMNPV DNA, as described in the '032 application (page 7, line 2, through page 8, line 4). The tested cells were HepG2, Sk-Hep-1, NIH3T3, NIH3T3 cells expressing a cell-surface asialoglycoprotein receptor, HeLa, CHO/dhfr$^-$, 293, COS, Ramos, Jurkat, HL60, K-562, C_2C_{12} myoblasts, C_2C_{12} myotubes, primary human muscle myoblasts, Hep3B cells, FTO2B cells, Hepa1-6 cells, and NGF-differentiated PC12 cells. Exogenous gene expression was detected in 15 of the 19 tested cell types. These results are summarized in Table 1; details of these examples follow.

Growth of Cells: Conventional tissue culture methods were used to grow the mammalian cells (Freshney, 1987, Culture of Animal Cells: A Manual of Basic Techniques, 2nd ed., Alan R.

Liss, Inc. New York, NY). Briefly, the cells were grown as follows. HepG2 and Sk-Hep-1 cells were cultured in minimal essential medium as modified by Eagle (EMEM) containing 10% fetal bovine serum (FBS). NIH3T3, HeLa, 293, and COS cells were cultured in DMEM containing 10% FBS. CHO/dhfr$^-$ cells were cultured in MEM alpha containing 10% FBS. Ramos, Jurkat, HL60, and K-562 cells were cultured in RPMI 1640 medium containing 10% FBS. HL60 cells were induced to differentiate by culture in the same medium containing 0.5% dimethyl sulfoxide and 1 μM retinoic acid (Sigma). C_2C_{12} myoblasts were propagated in DMEM containing 20% FBS and differentiated to myotubes during culture in DMEM containing 10% horse serum. PC12 cells were propagated in DMEM containing 5% FBS and 10% horse serum, and were induced to differentiate during culture in DMEM containing 10% FBS, 5% horse serum, and 100 ng/ml nerve growth factor. All cells were seeded one day prior to infection with AcMNPV, and multiplicities of infection were calculated assuming a doubling in cell number during this time.

In the *in vitro* assays described below, standard conditions for infection utilized 2 x 10^6 cells and RSV-lacZ AcMNPV (described in the '032 application) at a multiplicity of infection (moi) of 15 or 125 as indicated. Adherent cell lines were seeded one day prior to infection. Cells were exposed to virus in 2 ml of medium for 90 minutes, and then the virus-containing medium was removed and replaced with fresh medium. Mock-infected cells were treated with 2 ml medium lacking the viral inoculum.

Detection of Infection and Gene Expression: Delivery of a virus to a cell and expression of the exogenous gene were monitored using standard techniques. Expression of the exogenous gene was detected by two independent means: (*i*) histochemical staining with X-gal and (*ii*) a colorimetric assay of ONPG hydrolysis. For histochemical staining, cells were fixed in 2% (w/v) formaldehyde-0.2% (v/v) paraformaldehyde in phosphate buffered saline (PBS) for 5 minutes. After several rinses with PBS, the cells were stained by the addition of 0.5 mg/ml of X-gal (BRL) in PBS for 2-4 hours at 37°C.

For some cell types, β-galactosidase enzymatic activity was also quantitated using a colorimetric assay of ONPG hydrolysis as follows (Norton et al., 1985, Molecular & Cellular Biology 5:281-290). Cell extracts were prepared at one day post-infection. Cell monolayers were rinsed three times with PBS, scraped from the dish, and collected by low-speed centrifugation. The cell pellets were resuspended in 25 mM Tris pH 7.4/0.1 mM EDTA and then subjected to three cycles of freezing in liquid nitrogen and thawing in a 37°C water bath. The extracts were then clarified by centrifugation at 14,000 x *g* for 5 minutes. Standard conditions for assaying β-galactosidase activity utilized 0.1 ml of cell extract, 0.8 ml of PM-2 buffer, and 0.2 ml of *o*-nitrophenyl-α-D-galactopyranoside (4 mg/ml) in PM-2 buffer for 10 minutes at 37°C (Norton et al., 1985, Mol. & Cell. Biol. 5:281-290). The reaction was stopped by the addition of 0.5 ml of 1 M sodium carbonate. The amount of substrate hydrolyzed was detected spectrophotometrically at 420 nm, and

β-galactosidase enzymatic activity was calculated with conventional methods (Norton et al., 1985, Mol. & Cell. Biol. 5:281-290). The assay was verified to be linear with respect to extract concentration and time. Extract protein concentrations were determined using the Coomassie Plus protein assay (Pierce) with bovine serum albumin as a standard, and the level of β-galactosidase activity was expressed as units of β-galactosidase activity per mg of protein.

Results: Using the more sensitive method, which involved X-gal histochemical staining, expression of an exogenous gene was detected in 15 of the 19 mammalian cell types that were tested (79%). These results are summarized in Table 1.

High levels of exogenous gene expression were detected in HepG2 cells, 293 kidney cells, and NGF-treated PC12 nerve cells. β-galactosidase activity was also detected in Hep3B cells treated with the virus; the level of expression in these cells was nearly equivalent to the level detected with HepG2 cells. In addition, β-galactosidase activity was found in FTO2B (rat hepatoma) cells and Hepa1-6 (human hepatoma) cells exposed to virus. β-galactosidase activity was also detected in NIH3T3 cells that were engineered to express the asialoglycoprotein receptor on the cell surface. These cells expressed approximately two times the level of β-galactosidase expressed by normal NIH3T3 cells.

TABLE 1. EXOGENOUS GENE EXPRESSION DETECTED IN 15 OF 19 MAMMALIAN CELL TYPES

Cell Type	Exogenous Gene Expression	Cell Type	Exogenous Gene Expression
HepG2	+ (15)	Hep3B	+ (15)
FTO2B	+ (15)	NIH3T3 (+ ASGPR)	+ (15)
293	+ (15)	Hepa1-6	+ (15)
PC12 (+NGF)	+ (15)	COS	+ (125)
HeLa	- (125)	CHO/dhfr$^-$	+ (125)
NIH3T3	+ (125)	Sk-Hep-1	+ (125)
C_2C_{12} myoblasts	+ (125)	C_2C_{12} myotubes	+ (125)
primary myoblasts	+ (125)	K-562	- (125)
HL60	- (125)	Jurkat	- (125)
Ramos	- (125)		

"+" Exogenous gene expression was detected at the tested moi (15 or 125, shown in parentheses).

"-" Exogenous gene expression was not detected at the tested moi (15 or 125, shown in parentheses).

These data also show that the level of exogenous gene expression can be increased by using a higher dosage of virus (i.e., moi). Thus, cells that, at a low moi of 15, might appear to be refractory to the virus do in fact express the exogenous gene when the virus is administered at a higher moi. Examples of cell types that were able to be transfected at a higher moi include SK-Hep-1, NIH3T3, HeLa, CHO/dhfr$^-$, Cos, C_2C_{12} myoblasts, C_2C_{12} myotubes, and primary human muscle myoblasts.

The effect of virus dosage can readily be seen with HepG2 cells. When HepG2 cells were exposed to the Z4 baculovirus at a

moi of 15, approximately 5-10% of the cells stained blue with X-gal. At a moi of 125, approximately 25-50% of the cells stained blue, indicating an increase in the level of exogenous gene expression. Similarly, when Sk-Hep-1 cells were exposed to virus at a moi of 125, several cells were found to express the exogenous gene after treatment with this higher dose of virus. These data indicate that cells that simply appear to be refractory to the virus at a relatively low moi can, in fact, be infected and express the exogenous gene at a higher dosage of virus. Stained cells were not found in mock-infected control cultures. In sum, the experiments described above provide 15 examples of exogenous gene expression using a baculovirus to infect a mammalian cell.

When a less sensitive assay, the quantitative, colorimetric assay of β-galactosidase activity, was used to test 14 of the mammalian cell types, 3 cell types showed statistically significant ($P<0.05$, Student's t-test) higher β-galactosidase activity at a moi of 15 (Table 2). These cell types were HepG2, 293, and PC12. The human liver tumor line HepG2 exposed to the RSV-*lacZ* baculovirus expressed greater than 80-fold higher levels of β-galactosidase than did mock-infected controls. The adenovirus-transformed human embryonal kidney cell line 293 expressed the *lacZ* reporter gene at a level that was approximately four-fold over background. PC12 cells, which were differentiated to a neuronal-like phenotype with nerve growth factor, exhibited approximately two-fold higher β-galactosidase

levels after infection with the RSV-*lacZ* baculovirus. This difference was statistically significant (P=0.019).

TABLE 2. B-GALACTOSIDASE LEVELS MEASURED IN A QUANTITATIVE COLORIMETRIC ASSAY FOR 3 MAMMALIAN CELL TYPES

	β-galactosidase activity (units/mg) Mean ± SD	
Cell Line	**Mock Infected**	**RSV-*lacZ* Virus**
HepG2	0.030±0.004	2.628±0.729
293	0.092±0.014	0.384±0.024
PC12 (+NGF)	0.019±0.005	0.033±0.004

In sum, this set of experiments shows that exogenous gene expression can be achieved in a wide variety of cell types at an moi of 15 or 125. These data confirm that, as was asserted in the application, the baculovirus can be used with numerous cell types in addition to liver cells.

II. Expression in Neuronal and Glial Cells of Rat Cerebral Cortex Cultures

In this set of experiments, two additional examples are provided to demonstrate that a baculovirus can be used to express an exogenous gene in cultured neuronal and glial cells. For this example, the Z4 virus was prepared from Sf9 cells grown in Hink's TNM-FH media containing 10% FCS, as described above. The virus was purified by banding on a 20-60% sucrose gradient in phosphate-buffered saline. The titer of the virus employed in the following experiments was 3 x 10^8 pfu/ml (for virus stock #1) or 2 x 10^9 pfu/ml (for virus stock # 2), as measured on Sf9

cells. Each virus stock was sonicated prior to use. In these examples, the moi ranged from 1 to 667, which fits within the range recommended in the specification (0.1-1,000).

Example 1: Cortex Cultures from E16 Rat Embryonic Pups

For the first example, rat cerebral cortex cultures were prepared from E16 embryonic pups. A 24-well dish was seeded with 300,000 cells/well, and, at 4 days post-plating, the cells were infected by adding varying amounts of virus in serum-containing medium to the wells, as is indicated in Table 3. The virus was allowed to adsorb onto the cells for 24 hours.

TABLE 3. EXPRESSION OF AN EXOGENOUS GENE IN RAT CORTICAL CELLS

VIRUS	1 µl	2 µl	5 µl	10 µl	50 µl	100 µl
Z4 Stock #1	moi = 1	moi = 2	moi = 5	moi = 10	moi = 50	moi = 100
	no blue cells	no blue cells	~5 blue cells	~20 blue cells	~500 blue cells	~2200 blue cells (~0.75%)
Z4 Stock #2	moi = 6.7	moi = 13.3	moi = 34	moi = 67	moi = 335	moi = 667
	few blue cells	~100 blue cells	~200 blue cells	~450 blue cells	~1000 blue cells	~1300 blue cells
PBS				no blue cells	no blue cells	no blue cells

Expression of the exogenous β-galactosidase gene was measured by counting the number of blue cells after staining the cells with X-gal. Table 3 provides the number of blue cells observed in five fields of the microscope at 10X magnification; each well contained approximately 65 fields.

These data indicate that the exogenous β-galactosidase gene was expressed from the virus in the cultured neuronal cells. In contrast, no blue cells were detected when the cell cultures were

mock-infected with PBS. Analysis of the blue cells using standard cell morphology criteria revealed that the blue stained cells included both neuronal and glial cells. Thus, this baculovirus can be used to express an exogenous gene in neuronal and glial cells.

Example 2: Cortex Cultures from E20 and P1 Rat Embryonic Pups

In the second example, the Z4 baculovirus was used to express an exogenous gene in cultured cortical cells obtained from rat pups at the E20 and P1 stages. The cells from E20 pups were plated in 24-well dishes at 380,000 cells/well. The cells from P1 pups were plated at 300,000 cells/well. The E20 cultures were treated with araC (to inhibit the growth of glia) at 6 days post-plating, and they were infected at 10 days post-plating. The P1 cultures were treated with araC at 2 days post-plating, and they were infected at 6 days post-plating. Samples of each culture were infected with various dilutions of Z4 virus stock that had a starting titer of 2×10^9 pfu/ml. For comparison, similar cells were also infected, in a separate experiment, with Herpes Simplex Virus (HSV) expressing the *lacZ* gene placed under the control of an RSV promoter. (HSV is known to infect mammalian neuronal cells.) The titer of this HSV stock was 2×10^7 IU/ml, as measured on PC12 cells with X-gal histochemistry. For a negative control, the cells were mock-infected with PBS. Expression of the exogenous *lacZ* gene was measured by counting the number of blue cells obtained upon staining the cells with X-gal.

The Z4 virus of the invention successfully expressed the exogenous *lacZ* gene in cultured cortical cells obtained from rat pups at both the E20 and P1 stages of development. With 1-100 μl of the Z4 virus, 4.9-10% of the cortical cells at the E20 stage and 2.1-5.75% of the cortical cells at the P1 stage were stained blue with X-gal, indicating that the exogenous gene was expressed in those cells. Of the cells infected with 0.1-5.0 μl of the HSV RSVlacZ virus (the positive control), 1.9-3.4% of the E20 cells and 0.45-4.2% of the P1 cells stained blue with X-gal. When E20 and P1 cortical cells were mock-infected with PBS, as a negative control, no blue cells were detected.

These data provide additional evidence that the baculoviruses can be used to express an exogenous gene in mammalian cells other than liver cells. These data also indicate that the level of expression that can be obtained with the Z4 virus is comparable to the level of expression obtained with HSV, a virus known to infect mammalian cells.

III. Examples of the Use of Baculovirus to Express an Exogenous Gene in a Variety of Mammalian Cell Types *In Vivo*

This set of experiments provides examples in which the Z4 baculovirus was used to express an exogenous gene in mammalian cells *in vivo*. In one example, expression of the exogenous gene was detected in skin after topical application of the Z4 virus to abraded skin. In the remaining examples, exogenous gene expression was detected after a baculovirus carrying an exogenous gene was injected directly into one of the following tissues:

skin, spleen, kidney, stomach, skeletal muscle, uterus, and pancreas.

Example 1: Expression in Skin Following
Topical Application of Baculovirus to Abraded Skin

This example demonstrates that topical application of the Z4 virus to abraded skin of a mouse can result in expression of an exogenous gene in the skin tissue. Topical application of 50 μl of the Z4 virus to 1 cm^2 of abraded skin of a mouse resulted in expression of the exogenous gene in nearly 100% of the cells of the basal layer of the epidermis. These experiments involved four regions of the skin of a mouse. In one region, cells in various areas of the epidermis were stained blue by X-gal, indicating that the exogenous gene was expressed in the skin. In a second region, occasional blue-stained cells were present. In a third region, patches of blue-stained cells were detected. In a fourth region, the pattern of blue-stained cells was nearly continuous and the cells were a very dark blue, indicating high levels of exogenous gene expression. Although the pattern of gene expression varied among the four regions of skin in the experiment, this example demonstrates that topical application of the Z4 virus to abraded skin consistently resulted in expression of the heterologous gene in skin tissue.

As a negative control, PBS was applied onto abraded skin. An insubstantial level of blue-stained cells was detected, and it was limited to the sebaceous glands, not the epidermis. In addition, the staining was qualitatively different from the

staining observed in the presence of baculovirus. When PBS was administered, a punctate, rather than dispersed, pattern of staining was detected. The quantity and quality of staining observed with PBS suggests that staining of the negative control cells was due to the presence of bacteria on the skin of the mouse. Similar results were obtained when the Z4 virus was applied topically to unabraded skin. In sum, treatment of abraded skin cells with the Z4 virus resulted in exogenous gene expression in approximately 100% of the cells of the basal layer of the epidermis, while no significant staining was detected in control cells. These data therefore demonstrate that a baculovirus can be used to express an exogenous gene in skin cells.

Examples 2-8: Expression in Spleen, Kidney, Skin, Stomach, Skeletal Muscle, Uterus, and Pancreas

In these examples, expression of an exogenous gene was detected *in vivo* after a baculovirus carrying the gene was injected directly into seven different tissues. These tissues included spleen, kidney, skin, stomach, skeletal muscle, uterus, and pancreatic tissues. In these examples, the titer of the Z4 virus, as determined in a conventional plaque assay, was 4.8×10^{10} pfu/ml.

To assay for gene expression in spleen, kidney, muscle, or skin, the Z4 virus was administered to Balb/c female mice by direct injection of a 50 μl aliquot of the virus (2.4×10^9 pfu total) into the target organ. To spread the virus throughout an

organ, the 50 μl virus sample was injected into two or three sites of the organ. For assaying gene expression in the spleen, an uninjected mouse served as a negative control. For assaying gene expression in kidney, muscle, and skin, contralateral controls were performed (the Z4 virus was injected into the organ on the right side of the mouse, and 50 μl PBS was injected into the organ on the left side of the mouse). For assaying expression in muscle, the virus was injected into the tibealis anterior hind leg muscle after shaving the mouse. For assaying expression in skin, the abdomen of the mouse was shaved, and 50 μl of Z4 virus were injected into a marked section of the abdomen.

At 24 hours post-injection, the mice were sacrificed and dissected. The Z4- and PBS-injected organs were frozen in liquid nitrogen, and 7 μm thin sections were prepared using a cryostat (Reichert-Jung Cryocut 2800). β-galactosidase activity was measured by fixing the thin sections and staining with X-gal, using standard techniques. As described below, each of the organs that received the Z4 virus expressed the exogenous *lacZ* gene *in vivo*. In each case, the PBS negative control did not lead to expression of the exogenous gene.

Expression Following Injection of Virus Into Skin: In this example, *in vivo* expression of the exogenous *lacZ* gene of Z4 again was detected in mouse skin. In this example, 2.4×10^9 pfu of virus was injected into the skin, rather than topically applying the virus to abraded skin. Over 50% of the cells near the injection site expressed the exogenous *lacZ* gene, and *lacZ*

expression was detected throughout several cell layers of the epidermis. More than 25% of the cells in the dermis expressed the exogenous gene after subcutaneous injection of the virus. Positive staining of some skeletal muscle fibers also was detected. These data indicate that the Z4 virus can be used to express a heterologous gene in the skin of a mammal.

Expression in Spleen: In this example, thin sections of the spleen were assayed for gene expression following injection of the virus carrying the exogenous gene into the spleen. Expression of the *lacZ* gene was detected in cells scattered throughout the spleen, indicating significant expression of the exogenous gene. No blue coloration was detected in the control spleen that was injected with 50 μl of PBS. These data thus indicate that an exogenous gene can be expressed in a spleen cell *in vivo* following administration injection of a baculovirus genome that carries the exogenous gene.

Expression in Kidney: In this example, *in vivo* expression of an exogenous gene was detected in kidney tissue that was injected with Z4 as described above. The Z4-injected kidney displayed blue coloring that is indicative of *lacZ* expression; in contrast, a PBS-injected control kidney displayed no blue coloration. This example provides an additional demonstration that a baculovirus can be used to express an exogenous gene in tissues other than liver tissue.

Expression in Stomach: In this example, the Z4 virus (50 μl) was injected into the center of the stomach wall of Balb/C mice. The animals were sacrificed on the day following

injection, the stomachs were frozen in liquid nitrogen, and then cryostat sectioned and stained using conventional methods. Exogenous gene expression was detected in gastric mucosal and muscle cells. Positive staining was also detected in glands, with most staining occurring at the bases of the glands. Thus, a baculovirus virus can be used to express a heterologous gene in stomach tissue.

Expression in Skeletal Muscle: In this example, *in vivo* expression of the exogenous *lacZ* gene of Z4 was detected in muscle tissue after direct injection of virus into the tibialis anterior. Significant blue coloration was found in discrete loci in the muscle, indicating that a baculovirus can be used to express an exogenous gene in muscle tissue.

Expression in Uterus: In this example, expression of the *lacZ* reporter gene was detected in cells of the uterus. In this example, 50 μl of the Z4 virus (2.4 x 10^9 pfu) were injected directly into the uterus of a mouse. The animal was sacrificed on the day following injection, and cryostat sections were prepared using standard methods. Staining of the sections with X-gal produced blue coloration in cells of the uterus, particularly in endometrial stromal cells. Thus, this example provides an additional demonstration that a baculovirus can be used to express an exogenous gene in a variety of cell types.

Expression in Pancreas: This example demonstrates that a baculovirus can be used to express a heterologous gene in pancreatic cells. A 50 μl aliquot of the Z4 virus (2.4 x 10^9 pfu) was injected directly into the pancreas of a mouse. On the

day following injection, the mouse was sacrificed, and the pancreas was stained with X-gal according to conventional methods. Large areas of blue-stained cells were detected, indicating that the Z4 virus successfully expressed the *lacZ* gene in the pancreas.

IV. Expression of a Therapeutic Gene in Human Cells

In this example, a baculovirus was used to express ornithine transcarbamylase (OTC) under the control of a cytomegalovirus immediate early 1 (CMV IE1) promoter in human liver cells (see page 18, line 3, of the specification). In humans, a deficiency in OTC expression in the liver results in a disorder of the urea cycle (see, e.g., page 13 of the specification). Thus, expression of OTC in the liver can ameliorate this gene deficiency disorder. The details of the experiment are described below.

Human HepG2 cells were infected at a multiplicity of infection (moi) of 100 with various recombinant baculoviruses. The cells were exposed to the virus for 2 hours in serum-free medium (EMEM, 1 ml in a 60 mm petri dish). Medium containing serum (3 ml EMEM with 10% cosmic calf serum) was then added. At 24 hours post-infection, the cells were rinsed, scraped from the dish using a plastic policeman, and pelleted, and the total cellular protein was resuspended in sample buffer. The samples were subjected to SDS-PAGe, transferred to a nylon membrane, and probed with a rabbit polyclonal antibody to human OTC

(at 1:2,000). This Western blot was developed using chemiluminescence (ECL kit from Amersham).

The results of this Western blot are shown in the accompanying Fig. 1, and the contents of the samples in each lane are described below:

Lane	Sample
1	Mock-infected cells.
2	CMV-OTC1 virus (in this virus, the human OTC gene is placed under the control of a CMV IE1 promoter.
3	CMV-OTC2 virus (in this virus, the human OTC gene is placed under the control of a CMV IE1 promoter, and the virus contains a Kozak consensus sequence for translation, rather than the normal OTC translation start site.
4	CMV-lacZ virus alone.
5	Mouse liver, total cellular protein.

The samples in lanes 1 and 4 are negative controls and show no expression of OTC. The sample in lane 5 is a positive control and shows the expected pattern of OTC expression. A prominent species having a molecular weight of approximately 36 kD is the mature form of the protein (arrow). A smaller amount of the OTC precursor protein of approximately 40 kD is also detected; this precursor contains a signal peptide that is removed upon import into the mitochondria. The samples in lanes 2 and 3 both show expression of the mature form (36 kD) of OTC, indicating that the proper process occurs after expression of human OTC from the baculovirus vectors. This Western blot clearly shows that substantial amounts of human OTC are produced with the

baculoviral expression system. Levels of hOTC of 10% of normal levels, or even less, are expected to yield therapeutic benefit; therefore, the levels of gene expression obtained with the claimed baculoviral expression can be expected to confer therapeutic benefit to a patient suffering from a gene deficiency disorder. When these data are viewed in combination with the above-described examples of exogenous gene expression *in vivo*, expression of exogenous therapeutic genes *in vivo* can be expected to be successful for treating a gene deficiency disorder.

IV. Summary

In sum, these experiments demonstrate that a baculovirus can be used to express an exogenous gene in a variety of mammalian cell types *in vitro* or *in vivo*. Exogenous gene expression was detected in nearly all of the tested cell types, including: HepG2, 293, PC12 (+NGF), HeLa, CHO/dhfr$^-$, C_2C_{12} myoblasts, C_2C_{12} myotubes, primary human myoblasts, Sk-Hep-1, NIH3T3, NIH3T3 (expressing an asialoglycoprotein receptor), Hep3B, FTO2B, Hepa 1-6 cells, neurons, cells from the glia, skin, spleen, kidney, stomach, skeletal muscle, uterus, and pancreas. These examples also demonstrate that many cell types which at a low moi might appear to be refractive to baculoviruses can successfully be transfected simply by increasing the dosage of virus. In all of the examples summarized herein, the dosage of virus was within the range taught in the specification (see pg. 16, line 13). Furthermore, these data show that the claimed baculoviruses can be used to express a therapeutic gene in human cells, and the

level of gene expression is substantially high, such that a therapeutic benefit can be expected to be conferred upon a mammal containing the cells.

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: October 8, 1997 Frederick M Boyce MD PhD

Frederick M. Boyce, M.D., Ph.D.

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